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On the Mechanisms of Oligopeptide Reactions in Solution and Clay Dispersion

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Abstract: Mechanisms of the reactions of representative dipeptides (Gly_2, Gly-Ala), oligopeptides (Gly_3, Gly_4) and the polypeptide (poly-$Gly)_n$) in solution and clay suspensions at 85 °C were investigated. The reaction products and their yields were analysed and determined by means of HPLC. Interestingly, hydrolysis, where water molecules act as the reactant, was not the main reaction, even for oligopeptides. Formation of cyclic dipeptides prevailed in the reactions of dimers as well as oligopeptides. The breakdown of oligopeptide molecules proceeded via an intramolecular cyclization reaction. For example, the reaction of Gly_3 led to the formation of equal amounts of cyclic dipeptide, $c(Gly)_2$ and Gly. The presence of clay (montmorillonite) significantly increased yields in the reactions of dipeptides but it did not have much effect on the reactions of oligopeptides. However, an opposite effect of clay, protection of poly$(Gly)_n$ against decomposition, was proven.

Keywords: cyclic dipeptide formation; montmorillonite; oligopeptides; peptide bond; polyglycine

INTRODUCTION

The relevance of inorganic substances, such as minerals and salts, for the prebiotic formation of peptides has been summarized in several reviews, e.g. [1–5]. The hypothesis of formation of the first biopolymers in environments of ancient seas and oceans is not supported by experiments. Biopolymers, such as proteins and nucleic acids, are formed from their monomer units by condensation reactions, where water molecules are a side product of the reaction. Hydrolysis, which is a backward and main competitive reaction to condensation, prevails under the conditions of dilute aqueous solutions. Realistic scenarios of prebiotic formation of biopolymers cannot assume the presence of activated reactants or energy-rich compounds, which would enhance the reaction yields in seas and oceans. If such compounds had ever been formed in the primitive ocean, it could have been only in limited amounts and for a very short time scale due to their sensitivity to hydrolysis [6]. The more realistic theories consider the formation of the first biopolymers in environments where the concentration of the reactants could have been enhanced. Such sites would include small lakes or lagoons, where the concentrations may change in a periodic manner by drying/wetting processes [7]. A few experiments already confirmed that fluctuation conditions are more favourable for the formation of oligopeptides [8].

One of the most realistic theories of the prebiotic formation of short oligopeptides from amino acids considers the catalytic role of transitional metal

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complexes, mainly those of Cu(II). Experimental studies confirmed the ability of Cu(II) in sodium chloride solution to catalyse peptide bond formation from a variety of amino acids, preferring α-isomers [2]. Significant yields of Cu(II)-catalysed reactions are mostly restricted to small oligopeptides [3]. Many researchers assume that longer oligopeptides could have been formed on the surface of catalytically active solid substances. The chief candidates of such solid catalysts are minerals. Indeed, several experiments have confirmed the catalytic efficiency of clay minerals [3], silica [9,10] and aluminium oxides [9] and detailed studies on the mechanisms of the peptide bond formation have been carried out. When starting with amino acids or dipeptides, several types of reaction mechanisms were identified. The reaction starting from one or two reactants, amino acid(s) and/or dipeptide(s), is in principle a very complicated process [8], and in general includes amino acid dimerization, cyclic dipeptide (or 2,5-dioxopiperazine) formation [11] and peptide chain elongation [12]. Further reactions are hydrolysis, sequence inversion of dipeptides, which proceeds via cyclic dipeptide [8], and possible decomposition reactions, such as deamination, decarboxylation, oxidation, etc. The reactions with fluctuating conditions are not very suitable for a detailed understanding of the reaction mechanisms, because the reaction conditions, which affect reaction kinetics as well as thermodynamics, change in a cyclic manner. The objective of this work was to study the reactions of some simple di- and oligopeptides in solution and clay dispersion under constant conditions. Since the reactions investigated here proceeded in aqueous environment, the hydrolysis of peptide bond was expected to be the dominating reaction. Surprisingly, the pathways of the main reactions were different and revealed a marginal role for the hydrolysis under the given conditions.

MATERIALS AND METHODS

Montmorillonite from Apache County, Arizona (SAz-1) was purchased from Source Clays, Clay Mineral Society, Columbia, University of Missouri. Natural material was first treated with hydrogen peroxide solution in order to remove any potential organic impurity. Then, it was purified by wet sedimentation, separating a 2 μm fraction from mineral admixtures. The purity of the mineral was confirmed by x-ray diffraction measurement and infrared spectroscopy. Montmorillonite was repeatedly saturated with calcium chloride solution (1 mol dm^{-3}) in order to prepare the Ca^{2+}-ion-exchanged form, which was eventually used in the experiment. An excess of calcium chloride salt was removed by repeated washing of suspension in deionized water and dialysis.

Amino acids, dipeptides, cyclic dipeptides and oligopeptides used as reactants as well as reference substances for analysis were purchased from Sigma (Taufkirchen, Germany), Bachem (Bubendorf, Switzerland) and Senn Chemical (Dielsdorf, Switzerland). L-Optical isomers of Ala itself and Ala-containing compounds were used. Poly$(Gly)_n$ of average relative molecular weight 5000 was purchased from Sigma.

10 mg of montmorillonite was mixed in glass vials with 1 ml of a 10 mM di- or oligopeptide (Gly_2, Gly_3, Gly_4, Gly-Ala) solution. The contents were mixed by ultrasonication. Vials were sealed and heated at 85 °C for 1–5 days. For the reaction of poly$(Gly)_n$, 10 mg of montmorillonite were mixed in glass vials with 5 mg of polypeptide and 1 ml of deionized water. The suspensions of poly$(Gly)_n$ and montmorillonite were treated in the same way as described above. Then, the supernatants were isolated from the suspensions by filtration through a 0.2 μm pore membrane. All supernatants were collected and stored in a freezing box before analysis.

All solutions were analysed with an Agilent 1100 series LC system using an Agilent Hypersil (ODS 5 μm/200 × 2.1 mm) column. The mobile phase composition was 10 mM sodium hexylsulfonate acidified with phosphoric acid to pH 2.5. The flow rate and temperature were 0.55 cm^3 min^{-1} and 35 °C, respectively. Ion-pairing reverse-phase chromatography allowed for good separation and analysis of all compounds with the exception of traces of some reaction products of poly-$(Gly)_n$, which were eluted at very short retention times. However, these compounds, attributed to impurities and/or reaction products of low molecular weight, did not interfere with the main products (oligopeptides) eluted at longer retention times. Detection was performed with a diode array detector at 195 nm. The reaction products were identified by the retention times and UV absorption spectra of authentic reference substances. The reaction yields were determined as a percentage of the reactant converted to the reaction product.

RESULTS AND DISCUSSION

The reactions of two dipeptides Gly_2 and Gly-Ala were initially investigated. Analysis of the reaction

systems detected several reaction products: in the case of the reaction of Gly_2, both peptide bond formation and hydrolysis proceeded. However, longer linear peptides were not detected. The conditions in dilute solution prevent condensation of two Gly_2 molecules. The amide bond was formed by intramolecular reaction, which led to the formation of cyclic dipeptide, $c(Gly)_2$ (Figure 1). Because of the intramolecular mechanism, cyclic dipeptide formation is independent of the concentration of the reactant molecules. The hydrolytic reaction led to the formation of small amounts of Gly. Interestingly, hydrolysis was not observed for the Gly-Ala system, i.e. the reaction of Gly-Ala did not lead to detectable yields of Ala or Gly (Figure 2). However, one should point out a lower sensitivity of the analytical method used to detect amino acids. Nevertheless, the lower tendency of Gly-Ala to hydrolyse with respect to Gly_2 may be due to a larger energetic barrier for the hydrolysis reaction of Gly-Ala. The larger extent of the hydrolysis of Gly_2, in turn, may be due to the higher reactivities of Gly and its oligopeptides in general [1]. As Gly-Ala does not readily hydrolyse, it is also not readily formed from amino acids in the opposite reaction under similar conditions. Gly-Ala is formed in relatively low yields, but only in the reactions with fluctuation conditions and in the presence of catalysts, such as clays [8,13], inorganic oxides [8,14] or Cu(II)/NaCl [1,15]. Formation at temperatures below 100 °C in reactions without any catalysts has not been observed [8]. Another reason for the lower sensitivity of Gly-Ala to hydrolysis could be due to different properties of Ala. The methyl group in Ala stabilizes the amide group in the dipeptide via a positive inductive effect, and the higher electron density at the carbonyl carbon of the amide group may contribute to the lower reactivity.

Figure 1 shows yields of the reaction of Gly_2 in the presence and absence of clay. For the reaction without clay, the amount of Gly_2 dropped to about 92% of an initial concentration after 5 days. Almost twice more Gly_2 converted to the reaction products in clay suspension than in the absence of clay. Gly cyclic dipeptide formation exhibited higher yields than hydrolysis. After 5 days only 1% of Gly_2 solution hydrolysed to Gly. The presence of clay increased the hydrolysis yields almost twice. As for Gly cyclic dipeptide formation, the reaction yields were about 3% and 5%, respectively. Reaction rate constants were estimated considering two parallel reactions (Gly cyclic dipeptide formation and hydrolysis to Gly) considering reaction kinetics of first or pseudo-first order. In the absence of clay the

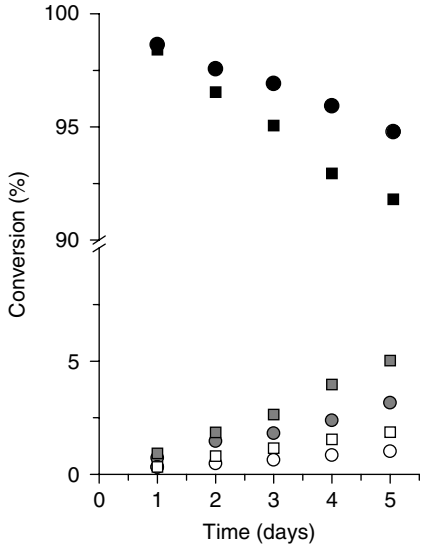

Figure 1 Formation of Gly cyclic dipeptide (grey) and glycine (open) from Gly_2 (solid) in the absence (circles) or presence of montmorillonite (squares).

rate constants of $c(Gly)_2$ and Gly formation were 7.13×10^{-8} s^{-1} and 2.25×10^{-8} s^{-1}, respectively. Considering that one Gly_2 molecule forms two Gly molecules, the above constants are in good agreement with the overall rate constant calculated for Gly_2 conversion from its concentration decrease with time (1.18×10^{-7} s^{-1}). The presence of clay increased the reaction rates to 2.13×10^{-7} s^{-1} [1.19×10^{-7} s^{-1} and 4.45×10^{-8} s^{-1} for $c(Gly)_2$ and Gly, respectively].

Interestingly, higher yields of cyclic dipeptide formation (8%–12%) were obtained from Gly-Ala (Figure 2). There are two possible explanations: (1) The reactivity of Gly-Ala for intramolecular cyclization reaction is higher than that of Gly_2. (2) The cyclic dipeptide, which contains an Ala unit in its molecule, is more stable against hydrolysis. Apparently, c(Ala-Gly) is more hydrophobic and would be less reactive than $c(Gly)_2$. Hence, the lower yields of Gly cyclization reactions (Figure 1) could be explained in terms of the hydrolysis of the reaction product back to the reactant. One should also note that the presence of clay increases the cyclization reaction significantly for both reaction systems, but has no significant effect on the sequence inversion reaction of Gly-Ala, i.e. the formation of Ala-Gly by hydrolysis of c(Ala-Gly). The explanation of this observation can be seen in the lower polarity of the molecules of cyclic dipeptide compounds and their negligible interaction with the polar clay surface. On the other hand, amino acids and oligopeptides

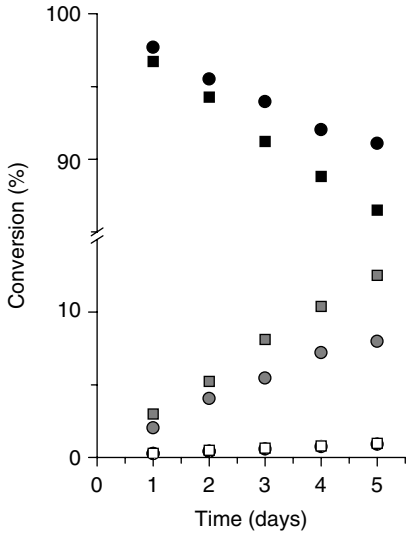

Figure 2 Formation of Gly-Ala cyclic dipeptide (grey) and Ala-Gly (open) from Gly-Ala (solid) in the absence (circles) or presence of montmorillonite (squares).

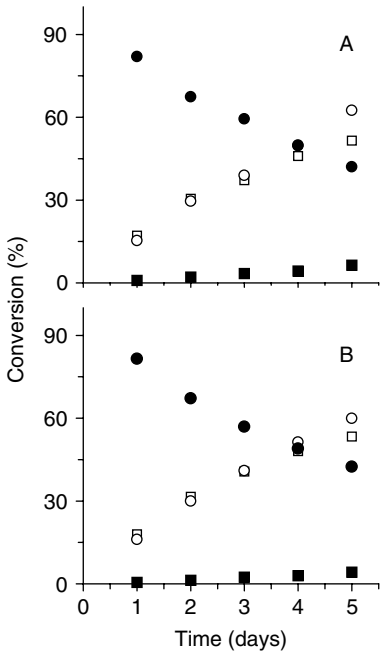

Figure 3 Formation of Gly cyclic dipeptide (open squares), glycine (open circles) and Gly_2 (solid squares) from Gly_3 (solid circles) in the presence (A) or absence of montmorillonite (B).

in aqueous systems are present in their ionic or zwitterionic forms and interact with both the clay surface and its polar groups, as well as with exchangeable cations.

Conversion of Gly_3 led to the formation of Gly, Gly_2 and $c(Gly)_2$ (Figure 3). There was a negligible effect of the clay presence on reaction yields. One would expect the hydrolysis of Gly_3 to Gly_2 and Gly as the main reaction products. However, relatively very small yields of Gly_2 were detected compared with those of Gly and $c(Gly)_2$. One possibility which would explain this apparent contradiction is that the cyclization reaction followed the formation of Gly_2, converting most of the product to $c(Gly)_2$. However, there were no such high yields of cyclization observed for the reaction starting directly with Gly_2 (Figure 1). Another, more plausible explanation is that the main reaction products, Gly and $c(Gly)_2$, were produced already in the first step reaction. Such a reaction would not run as a typical hydrolysis reaction, where water molecules act as a second reactant. Instead, the tripeptide would decompose by intramolecular rearrangement, which is a condensation reaction, where an amino group reacts with a carbonyl carbon atom of the second amide group (Figure 4). The capability of the Gly_3 molecules to perform such reactions has been confirmed by molecular dynamics simulation of oligoglycines in water, shown as videoclip in the Supplementary Materials*. Similar reactions, but proceeding in an opposite direction, had already been observed and studied in detail [8,16] and were considered as the last step of an alternative mechanism of peptide chain elongation. In this reaction the cyclic dipeptide, initially formed from the linear dipeptide, reacts with a second dipeptide (or amino acid) molecule by ring-opening. Then,

* http://www.molvision.com (Video Clips, Supplementary Materials to Publications in Journals, No. 9)

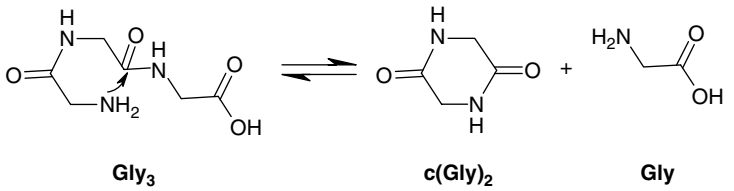

Figure 4 Scheme of the reaction mechanism of Gly_3.

a molecular rearrangement, via connection to the second reactant molecule, generates a longer oligopeptide chain [8]. A reaction of Gly_3, which would proceed under the same mechanism forming $c(Gly)_3$, would be theoretically possible, as well. However, we were unable to verify formation of $c(Gly)_3$. The sum of the reaction yields, approaching 100%, indicates that there is a low probability that $c(Gly_3)$ could have been formed in significant yields. Moreover, no peak in the chromatograms indicated formation of this compound (not shown). The absence of $c(Gly)_3$ could be explained in terms of a more favourable cyclization reaction producing a six-membered ring product, or in an alternative approach, of the lower reactivity of the charged COO^- groups (or of both). The latter assumption is supported by the comparison of the yields of Gly_2 (Figure 1) and Gly_3 (Figure 3) conversions. The cyclization reaction of Gly_2 afforded only about 5% of $c(Gly)_2$ in the presence of clay after 5 days, and the yield was even less for the reaction in solution. On the other hand, in the reaction of Gly_3, which is also a cyclization reaction, but proceeding at an amide carbon atom, the conversion reached about 53% under the same conditions (Figure 3).

In the reaction of Gly_3 we assume that Gly_2 was formed either in the second step from $c(Gly)_2$ or directly by hydrolysis of Gly_3. Nevertheless, the reaction yields of the products from the reaction of Gly_3 clearly show that the hydrolysis of Gly_3, where water molecules act as reactants, played a minor role compared with the cyclization reaction (Figure 4).

By taking into account various reaction mechanisms and the fact that the reaction products can further decompose, one would expect the interpretation of the reaction paths in the conversion of longer oligopeptides to be more difficult. However, the interpretation is possible, if one considers the reactions and the mechanisms observed for the shorter precursors, such as Gly_2 and Gly_3 (Figures 1 and 3). The same type of reaction mechanism, which was observed for the conversion of Gly_3, was expected for Gly_4 as well. However, there are two parallel reactions theoretically possible, which would proceed by the same mechanism, one at the carbon atom of the second amide bond and the other at the third amide bond. The first reaction would yield $c(Gly)_2$ and Gly_2. Indeed, both compounds were formed in similar reaction yields during the overall reaction time (Figure 5), which confirms the proposed reaction mechanism. Negligibly higher yields of $c(Gly)_2$

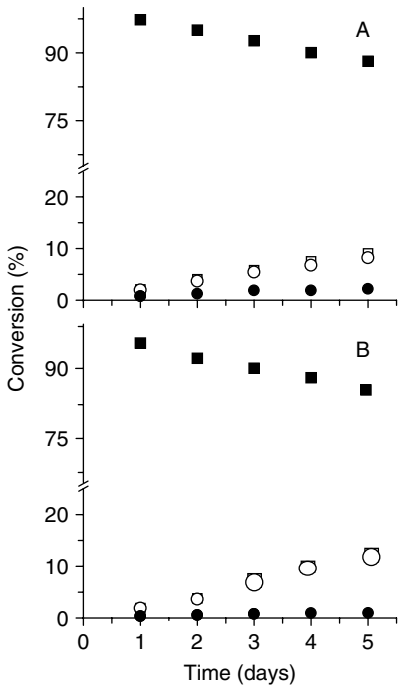

Figure 5 Formation of Gly cyclic dipeptide (open squares), Gly_2 (open circles) and Gly_3 (solid circles) from Gly_4 (solid squares) in the presence (A) or absence of montmorillonite (B).

can be interpreted in terms of the following conversion of Gly_2 to $c(Gly)_2$.

Besides Gly_2 and $c(Gly)_2$, Gly and Gly_3, were identified as other reaction products. However, they were probably formed directly by hydrolysis of Gly_4, since their yields were very low. Due to the lower sensitivity of the analytical method to amino acids, the yields of Gly could not be quantified, but merely estimated to be below 2%. Hence, the cyclization reaction at the third amide group, which would lead to the formation of equal amounts of $c(Gly)_3$ and Gly, was not observed. No unidentified peak(s) were found in the chromatograms which could have been assigned to $c(Gly)_3$ and would indicate its formation during the conversion of Gly_4.

Under conditions of low reactant concentrations, no higher peptides are formed from oligopeptides, either in solution, or in clay suspensions. Dipeptides yield mostly cyclic dipeptides. Oligopeptides, such as Gly_3 and Gly_4, also produce Gly cyclic dipeptide as the main product, and Gly or Gly_2, respectively, as remaining parts of the reactant molecules (Figures 3 and 5). In the reactions of dipeptides, Gly_2 and Gly-Ala, the reaction yields were strongly influenced by the presence of clay. For example, both the

hydrolysis and cyclization reactions of Gly_2 gave twice higher yields in clay suspensions than in pure aqueous solution (Figure 1). With increasing size of the oligopeptide molecule, the hydrolysis does not proceed to a large extent and the effect of the clay on the overall reaction yields is much lower. One may thus assume a role of clay minerals to stabilize oligopeptide molecules against decomposition via adsorption [17]. Under neutral pH conditions, the adsorption of amino acids, as well as dipeptides on clay minerals is very low [18]. For oligopeptides, adsorption increases with the chain length [19].

Another experiment was conducted to test the reaction of poly$(Gly)_n$ in water and clay dispersion. A variety of compounds were detected, as shown in the representative chromatograms of Figure 6. One has to consider the very low water-solubility of both the polypeptide and long-chain oligopeptides, which were formed as reaction products by hydrolysis. We identified and proved by reference substances the reaction products from Gly to Gly_6. Using an extrapolation of elution times, the identity of higher oligopeptides up to Gly_{11} was assumed [20]. Unknown reaction products were found at low retention times, which could be impurities or decomposition products of low molecular weight. The presence of these compounds somehow disturbs the detection of the Gly band. Only negligible amounts of c$(Gly)_2$ were determined, representing only 0.2% conversion of the total poly$(Gly)_n$ amount.

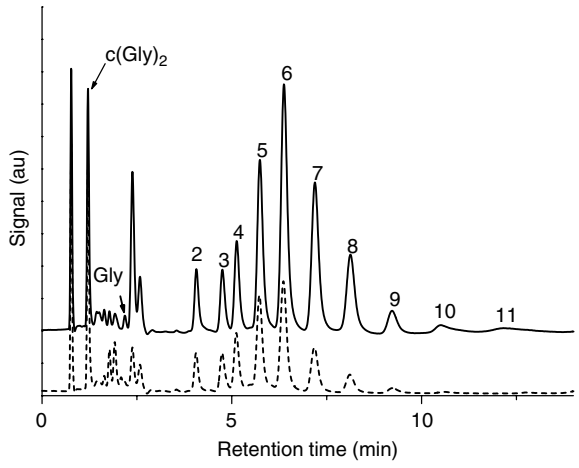

Figure 6 Chromatograms identifying reaction products and their relative amounts formed after 5 days by decomposition of poly$(Gly)_n$ in the absence (full) or presence of montmorillonite (dashed). Peaks, related to the elution of Gly_2 and oligopeptides, are identified by numbers, which denote the number of Gly units in the oligopeptide molecules.

The reaction yields of c$(Gly)_2$ were not much affected by the presence of clay. Reaction yields of any compound did not exceed 1%, the highest yield being found for Gly_2 formed without clay (0.85%). The amount of this compound in the reaction system including clay was significantly lower (0.50%). As clearly seen from the chromatogram in Figure 6, oligopeptides were formed and brought into solution in larger amounts in the reaction system without clay. For shorter chain oligopeptides this difference was not so high, but increased with the chain length, achieving more than twice higher amount for Gly_6. For longer oligopeptides, the difference was even more evident. In this case, the adsorption on the clay surface might reduce hydrolysis and/or the amount of the reaction products released into the solution. However, Gly_2 and the small oligopeptides are only negligibly adsorbed on a clay surface. The lower yields of Gly_2 and all oligopeptides produced from poly$(Gly)_n$ in clay suspension evidently prove an interesting ability of clay minerals to protect longer peptides against hydrolytic decomposition.

Acknowledgements

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